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14. ABSTRACT

The overall goal was to develop infectivity enhanced flt-1 promoter-based conditionally replicative adenovirus (CRAd) and investigates effects of CRAd virotherapy on breast cancer cells in vitro. We have developed a CRAd using the flt-1 promoter element for specific E1A gene expression (CRAdflt), RGD-4C-containing peptide in the HI loop of the Ad fiber knob region (CRAdRGDflt) and encoding the melanoma differentiation associated gene-7/interleukin-24 (mda-7/IL-24) gene (CRAdRGDflt-mda-7) and a RGD-4C modification in the fiber knob domain. In vitro experimental data provided evidence of relatively high levels of flt-1 promoter activity in human and mouse blood vessel endothelial cells and in certain human breast cancer cell lines. The comparative levels of CRAd replication in cancer cells correlated with flt-1 promoter activity. RGD-4C modification in the the fiber-knob region increased cytotoxicity of CRAdRGDflt in comparison with CRAdflt. The breast cancer cells demonstrated different levels of sensitivity to CRAdRGDflt-mda-7 infection. The induction of apoptosis in cancer cells after CRAdRGDflt-mda-7 infection was demonstrated.

15. SUBJECT TERMS
Breast cancer, conditionally replicative adenovirus, flt-1, melanoma differentiation associated gene-7/interleukin-24 (mda-7/IL-24), gene therapy

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INTRODUCTION

The objectives of this project are to develop infectivity enhanced flt-1 promoter-based conditionally replicating adenoviruses (CRAd) and investigate effects of CRAd virotherapy on endothelial cells and breast cancer cells *in vitro*. Vascular targeting exploits molecular differences between normal blood vessels and angiogenic tumor blood vessels. Primarily vascular endothelial growth factor (VEGF) receptor, flt-1, overexpression occurs in tumor vascular endothelium. Also, VEGF receptor is expressed by a variety of tumor cells including breast carcinoma. Importantly, the flt-1 promoter exhibits a "liver off" phenotype when used in adenoviral (Ad) vectors. A novel cancer growth-suppressing and apoptosis-inducing gene, the melanoma differentiation associated gene-7/interleukin-24 (*mda-7/IL-24*), has demonstrated profound and selective anticancer activity *in vitro* and *in vivo*, with minimal toxicity to normal cells. Additionally, secreted MDA-7/IL-24 has shown antiangiogenic activity.

CRAds using the flt-1 promoter element for specific *E1A* gene expression with wild type or RGD-4C modification in the HI loop of the Ad fiber-knob region and CRAd encoding the *mda-7/IL-24* gene have developed. The new vectors were evaluated *in vitro* for viral amplification, induction of apoptosis and breast cancer cell killing in serial comparative experiments. It was shown that the RGD-4C modified vectors demonstrated enhanced infectivity in comparison with wild type fiber CRAd. Specificity of flt-1 promoter for endothelial cells and breast cancer cells was demonstrated. The breast cancer cells demonstrated different sensitivity to CRAdRGDflt-mda-7 infection and levels of cytotoxicity and replication efficiency were correlated with flt-1 promoter activity. It was determined that CRAdRGDflt-mda-7 produced cell death was associated with induction of apoptosis in breast cancer cells.

BODY

Statement of Work (SOW) Task 2: To determine the relative Ad transductional efficacy and flt-1 promoter activity several human breast cancer cell lines were infected with Adflt-Luc or AdCMV-Luc replication-incompetent recombinant vectors. Comparing the flt-1 promoter with CMV promoter driven firefly luciferase reporter gene expression, specificity of flt-1 promoter for human and mouse blood vessel endothelial cells and several breast cancer cell lines was demonstrated (**Fig. 1A**). These data provide evidence of relatively high levels of flt-1 promoter activity in human endothelial cells and LCC6 breast cancer cells *in vitro*. As expected, the comparative levels of the Luc expression of breast cancer cells correlated with VEGF receptor mRNA expression when mRNA levels were assessed by RT-PCR (**Fig. 1B**).

SOW Task 1 and Task 2: To develop transcriptional and transductional targeted recombinant CRAd for breast cancer oncolysis. We have developed a CRAd using the flt-1 promoter element for specific *E1A* gene expression (CRAdflt). A major issue of Ad gene therapy is the fact that most tumor cells as well as endothelial cells demonstrate low levels of the coxsackie and adenovirus receptor (CAR) expression (**Table 1**). To address these limitations that may occur in primary breast cancer, we have developed retargeted CRAd employing the flt-1 promoter to control *E1A* gene expression with a CDCGRDCFC (RGD-4C) peptide inserted into the HI loop of the Ad fiber knob domain (CRAdRGDflt). To evaluate the biological activity of CRAdflt and CRAdRGDflt, we infected several breast cancer cell lines at various MOI (**Fig. 2**). The breast cancer cells demonstrated different levels of resistance to CRAdflt infection. There was a dose-dependent relationship in cell killing, measured by crystal violet staining assay. RGD-4C modification in the HI loop of the Ad fiber-knob domain enhanced cell death following CRAdRGDflt infection in comparison with CRAdflt (wild type Ad fiber knob). It is expected that CRAdRGDflt would be more effective against primary and metastatic breast cancer than CRAdflt.

We have developed a CRAd using the flt-1 promoter element for specific *E1A* gene expression and encoding the *mda-7/IL-24* gene (CRAdRGDflt-mda-7). Additionally, RDG-4C modification in the HI

loop of the Ad fiber-knob region was used for increasing efficiency of the Ad infection. In next set of studies, we determined the capacity of CRAdRGDflt-mda-7 virotherapy to induce breast cancer cell death. To verify the levels of MDA-7/IL-24 protein expression *in vitro*, human breast cancer cells were infected with 1 MOI of CRAdRGDflt-mda-7 or CRAdRGDflt (**Fig. 3**). As shown in **Fig. 4**, LCC6 cancer cells demonstrated relatively increased Ad fiber gene copy number in comparison with 2LMP cells, which correlated with higher levels of flt-1 driven Luc expression(**Fig. 1A**), mRNA levels (**Fig. 1B**), and replication efficiency in breast cancer cells (**Fig. 5**). Also, CRAdRGDflt-mda-7 demonstrated relatively high levels replication in human blood vessel endothelial cells (**Fig. 6**). To evaluate the biological activity of CRAdRGDflt-mda-7, we infected several breast cancer cell lines, MCF10A normal mammary gland epithelial, and BEAS-2B normal human bronchial epithelial cells with CRAdRGDflt-mda-7 or CRAdRGDflt at 1 MOI (**Fig. 7**). CRAdRGDflt-mda-7 produced increased breast cancer cell killing in comparison with normal human breast and bronchial epithelial cells in a dose-dependent manner (data not shown).

Table 1. Flow cytometry analysis of αvβ3 and αvβ5 integrins and CAR surface expression.

Cell line	**% of positive cells (mean of fluorescence intensity)***		
	αvβ3	**αvβ5**	**CAR**
2LMP	81±9 (30±12)	93±6 (55±10)	15±3 (18±5)
LCC6	96±6 (134±14)	92±7 (77±9)	17±2 (11±2)
MDA-MB-231	82±8 (39±2)	90±8 (158±25)	79±9 (54±8)
MDA-MB-468	14±2 (120±19)	91±7 (105±21)	11±4 (67±11)
MCF7	36±5 (70±11)	96±8 (131±27)	2±2 (23±7)
MCF10A	2±3 (30±5)	47±6 (38±4)	92±9 (120±15)
HEK293	89±10 (87±13)	94±7 (94±23)	97±8 (76±6)
HUVEC	91±8 (110±22)	90±9 (123±19)	83±11 (78±12)
PEA-10	71±8 (55±4)	49±6 (37±6)	11±2 (63±6)
SVEC 4-10	79±7 (36±6)	51±5 (34±6)	10±3 (20±5)
1P-1B	71±8 (34±8)	76±6 (115±17)	16±2 (45±11)

* Human breast cancer cells, MCF10A normal breast epithelial cells, human and mouse endothelial cells and HEK293 (as positive control for αvβ3 and αvβ5 integrins and CAR surface expression) were stained with saturating amounts of monoclonal antibodies recognizing αvβ3, αvβ5 or CAR and expression was evaluated by FACS analysis. A negative IgG isotype primary mouse antibody was used as a control. Data are the means ± SD of three independent experiments.

SOW Task 2: A series of studies were carried out to clarify the possible mechanisms underlying of CRAdRGDflt-mda-7-mediated cell death. To confirm that CRAdRGDflt-mda-7 oncolytic virotherapy induces apoptosis, breast cancer cells were stained with both propidium iodide (PI) and FITC-labeled annexin V. The cells were analyzed by flow cytometry (**Table 2**). The results indicate that CRAdRGDflt-mda-7 and CRAdRGDflt infection rapidly induced apoptosis and then necrosis at a later time.

To evaluate caspase activity after CRAdRGDflt-mda-7 infection Western blot analysis of cellular proteins using antibodies against caspases as well as PARP that can detect both unprocessed proenzyme and active form of proteins was performed. There was activation of caspase-3,-8 and -9 and cleavage of PARP in LCC6 breast cancer cells after CRAdRGDflt-mda-7 infection (**Fig. 8A**). Also, sensitivity of cancer cells to oncolytic virotherapy may be due to a balance of expression of pro-apoptotic and anti-apoptotic members of the *bcl-2* gene family, which are involved in regulation of *mda-7/IL-24*-induced apoptosis. Western blot analysis showed that the pro-apoptotic Bax protein is up-regulated after CRAdRGDflt-mda-7 in contrast with no effect to anti-apoptotic Bcl-2 and Bcl-xl expression (**Fig. 8A**). Additionally a time-dependent increase of caspases-3, -8 and -9 was demonstrated after infection of LCC6 breast cancer cells with CRAdRGDflt-mda-7, using Ac-DEVD-AFC, Ac-IETD-AFC and Ac-LEHD-AFC as substrates (**Fig. 8B**). Taken together, data shown in **Fig. 8** demonstrate that apoptosis induced by CRAdRGDflt-mda-7 infection in breast cancer cells is dependent on the activation of the caspase pathway.

Table 2. Apoptosis profile of breast cancer cells after CRAdRGDflt-mda-7 or CRAdRGDflt infection.*

Time	2LMP				LCC6			
(h)	AV-/PI-	AV+/PI-	AV+/PI+	AV-/PI+	AV-/PI-	AV+/PI-	AV+/PI+	AV-/PI+
				CRAdRGDflt				
0	97.1±6.2	2.4±1.1	0.4±0.5	0.1±0.3	96.0±5.6	3.1±0.9	0.4±0.3	0.5±0.4
16	88.3±6.9	8.1±3.2	1.9±0.3	1.7±0.3	81.4±6.5	15.3±2.7	2.1±0.5	1.2±0.4
24	85.5±7.1	10.3±2.6	1.8±0.4	2.4±0.4	75.1±5.8	18.1±2.1	4.5±0.7	2.3±0.7
36	82.2±5.8	12.4±2.1	2.7±0.4	2.8±0.4	67.8±6.0	22.2±2.5	5.4±0.7	4.6±0.5
48	79.3±6.1	14.6±1.9	2.6±0.5	3.6±0.6	61.2±5.9	26.6±3.0	7.0±0.6	4.2±0.5
				CRAdRGDflt-mda-7				
0	97.1±6.2	2.4±1.1	0.4±0.5	0.1±0.3	96.0±5.6	3.1±0.9	0.4±0.3	0.5±0.4
16	88.2±7.5	9.6±1.1	1.0±0.4	1.2±0.3	77.9±6.6	18.8±1.5	2.3±0.2	1.0±0.3
24	81.7±6.7	14.9±1.0	2.1±0.5	1.3±0.2	71.6±5.9	20.7±1.4	4.2±0.4	3.5±0.3
36	78.1±6.9	16.1±0.8	2.4±0.3	3.4±0.3	59.3±6.0	25.9±2.1	8.5±0.5	6.3±0.5
48	72.0±6.2	20.2±1.1	3.7±0.5	4.0±0.5	49.1±4.0	30.0±2.3	11.7±1.1	9.2±0.8

*For annexin V binding assay and PI uptake evaluation, after CRAdRGDflt-mda-7 or CRAdRGDflt infection at 1 MOI 2LMP and LCC6 breast cancer cells were collected over time as indicated and double stained with FITC-conjugated annexin V and PI. Samples were analyzed by FACS. Cells taking vital dye PI (AV+/PI+ and AV-/PI+) were considered necrotic, while annexin V-positive and PI-negative cells (AV+/PI-) were considered apoptotic, and percentages of cells were calculated. Data points are the mean values ± SD of two or three experiments, each performed in triplicate.

The results of these *in vitro* studies demonstrated that specific CRAdRGDflt-mda-7 oncolysis may be an effective approach for the treatment of breast cancers *in vivo*.

KEY RESEARCH ACCOMPLISHMENTS

- Developed new CRAd vectors encoding *E1A* under control flt-1 promoter element with wild type and RGD-4C modified HI loop Ad fiber-knob region and expressing *mda-7/IL-24* as therapeutic gene.
- Validated the flt-1 promoter activity in human breast cancer cells, murine and human blood vessels endothelial cells.
- Showed that RGD-4C modification of HI loop of Ad fiber-knob region increased CRAdRGDflt cytotoxicity of breast cancer cells in comparison with CRAdflt.
- Demonstrated cytotoxicity of breast cancer cells infected with flt-1 driven CRAd.
- Showed that CRAdRGDflt-mda-7 produced increased cytotoxicity of breast cancer cells in comparison with CRAdRGDflt.
- Validated the induction of apoptosis and expression of proteins involved in apoptosis regulation in cancer cells after infection with CRAdRGDflt-mda-7

REPORTABLE OUTCOMES

The results of this research were presented:

Kaliberov SA, Kaliberova LN, Petersen AS, Krendelchtchikova V, Krasnykh V, Buchsbaum DJ: Oncolytic virotherapy employing flt-1 driven mda-7/IL-24 gene delivery. The 8th Annual Meeting of the American Society of Gene Therapy, St. Louis, Missouri, June 1-5 2005.

Kaliberov SA, Kaliberova LN, Krendelchtchikova V, Petersen AS, Krasnykh V, Buchsbaum DJ: Angiogenesis-targeted conditionally replicating oncolytic adenovirus for breast cancer treatment. The 4th Era of Hope meeting for the Department of Defense (DOD) Breast Cancer Research Program (BCRP), Philadelphia, Pennsylvania, June 8-11 2005.

CONCLUSIONS

In conclusion, the combination of the transductional targeting using RGD-4C modification HI loop Ad fiber-knob region with transcriptional control of CRAd replication using the flt-1 promoter should improve specificity for CRAd oncolytic virotherapy. The most promising approach involves using the mda-7/IL-24 as therapeutic gene which increases CRAd produced cytotoxicity in breast cancer cells. The results provide the rationale to continue these investigations to achieve more effective treatment for breast cancer that can be pursued in future animal model.

ABBREVIATIONS

Ad, adenovirus;
CAR, coxsackie and adenovirus receptor;
CRAd, conditionally replicating adenovirus;
flt-1, VEGF receptor,
mda-7/IL-24, the melanoma differentiation associated gene-7/interleukin-24;
VEGF, vascular endothelial growth factor.

APPENDICES

[529] Oncolytic Virotherapy Employing flt-1 Driven mda-7/IL-24 Gene Delivery

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Vascular-targeted anticancer therapy using conditionally replicating adenoviruses (CRAd) is an attractive strategy for cancer treatment. For specific retargeting of CRAd, selective transcription of viral genes utilizing tumor-specific promoters has been developed. CRAd targeting using insertion of the tumor-specific binding RGD ligand in the Ad fiber has shown increased infectivity. A cancer growth-suppressing gene, *mda-7/IL-24*, has demonstrated profound and selective anticancer activity. The central hypothesis of this study was that selective transcriptional and transductional targeting of CRAd encoding the *mda-7/IL-24* gene using flt-1 promoter for specific *E1a* expression and RDG-4C modification of the Ad fiber-knob region can increase efficacy *via* direct killing of tumor endothelial cells and *flt-1*-positive cancer cells. Specific *mda-7/IL-24* overexpression should enhance the anti-tumor efficacy of CRAd oncolytic virotherapy alone. The flt-1 promoter-driven firefly luciferase expression was determined using replication-defective Adflt-Luc and AdCMV-Luc vectors on a panel of human glioma, breast, ovarian and prostate cancer cells and human or mouse endothelial cells. The cytotoxicity of cancer and endothelial cells to CRAd infection was analyzed using the crystal violet inclusion assay. The CRAd progeny production was determined using real time PCR and a standard 50% tissue culture infectious dose assay. Tumor xenograft models were used to determinate the therapeutic efficacy of oncolytic virotherapy to treat human glioma, breast, ovarian and prostate tumors. Specificity of flt-1 promoter for human and mouse endothelial cells and U251MG glioma, PA-1 ovarian, LCC6 breast, and DU145 prostate cancer cells was demonstrated. We have developed a CRAd using the flt-1 promoter element for specific *E1a* gene expression, encoding the *mda-7/IL-24* gene (CRAdRGDflt-mda-7) and a CDCGRDCFC-containing peptide in the HI loop of the fiber knob domain. The cancer cells demonstrated different levels of cytotoxicity after CRAdRGDflt-mda-7 infection. There was a significant decrease in viability of U251MG, PA-1, LCC6, and DU145 cells infected with CRAdRGDflt-mda-7. *In vivo* therapy studies demonstrated that intratumoral injection of CRAdRGDflt-mda-7 produced significant inhibition of subcutaneous LCC6 breast, OVCAR-3 ovarian, and DU145 prostate cancer xenografts and delay of D54MG human glioma tumor growth in athymic nude mice. These results suggest that selective targeting of oncolytic virotherapy with enhanced potency via mda-7/IL-24 pro-apoptotic/anti-angiogenic gene expression would be a superior strategy for treatment of human cancer.

This work was supported in part by NCI SPORE in Brain Cancer grant # P50 CA097247 and the USAMRMC under W81XWH-04-1-0663.

P46-7: ANGIOGENESIS-TARGETED CONDITIONALLY REPLICATING ONCOLYTIC ADENOVIRUS FOR BREAST CANCER TREATMENT

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Abstract

High levels of vascular endothelial growth factor (VEGF) production by breast cancer cells and robust expression of its cognate flt-1 receptor in tumor-associated blood microvessels suggest that VEGF/VEGF receptor expression plays an important role in breast cancer angiogenesis. Vascular-targeted anticancer therapy using conditionally replicating adenoviruses (CRAd) that specifically infect and propagate in tumor blood vessel endothelial cells is an attractive strategy for breast carcinoma. For specific retargeting of CRAd, selective transcription of viral genes utilizing tumor-specific promoters has been developed. Additionally, transductional CRAd targeting using insertion of the tumor-specific binding Arg-Gly-Asp (RGD) ligand in the Ad fiber has shown promise. To enhance the antitumor efficacy, several CRAds have been engineered to function as therapeutic gene delivery vehicles. A novel cancer growth-suppressing and apoptosis-inducing gene, *mda-7/IL-24*, has demonstrated profound and selective anticancer activity, with minimal toxicity to normal cells. The central hypothesis of this proposal was that selective transcriptional and transductional targeting of CRAd encoding the *mda-7/IL-24* gene using flt-1 promoter for specific *E1a* expression and RDG-4C modification in the HI loop of the Ad fiber-knob region can increase efficiency and specificity of breast cancer therapy via direct killing of tumor endothelial cells and *flt-1*-positive breast cancer cells. Moreover, specific MDA-7 protein overexpression should enhance the antitumor efficacy of CRAd virotherapy against breast carcinoma cells.

Specificity of flt-1 promoter-driven firefly luciferase (Luc) expression was determined using Adflt-Luc and AdCMV-Luc replication-incompetent Ads on a panel of human breast cancer and human or mouse endothelial cells. The flt-1 and GAPDH mRNA levels were assessed by RT-PCR. The sensitivity of breast cancer and blood vessel endothelial cells to CRAd infection was analyzed using the crystal violet inclusion assay. The CRAd progeny production was determined using real time PCR and a standard 50% tissue culture infectious dose assay.

Specificity of flt-1 promoter for endothelial cells and breast cancer cells was demonstrated. The comparative levels of Luc expression of breast cancer cells correlated with VEGF receptor mRNA expression. We have developed a CRAd using the flt-1 promoter element for specific *E1a* gene expression, encoding the *mda-7/IL-24* gene (CRAdRGDflt-mda-7) and a CDCGRDCFC-containing peptide in the HI loop of the fiber knob domain. The breast cancer cells demonstrated different levels of sensitivity to CRAdRGDflt-mda-7 infection. There was a significant decrease in cell viability of 2LMP and LCC6 breast cancer cells infected with CRAdRGDflt-mda-7 as compared with Adflt-Luc-infected cells.

These results suggest that selective targeting of virotherapy with enhanced potency via *mda-7/IL-24* pro-apoptotic/anti-angiogenic gene expression would be a superior strategy for treatment of breast cancer. The results provide the rationale to continue these investigations to achieve more effective treatment for breast cancer that can be pursued in future animal model and human clinical trials.

The U.S. Army Medical Research and Materiel Command under W81XWH-04-1-0663 supported this work.

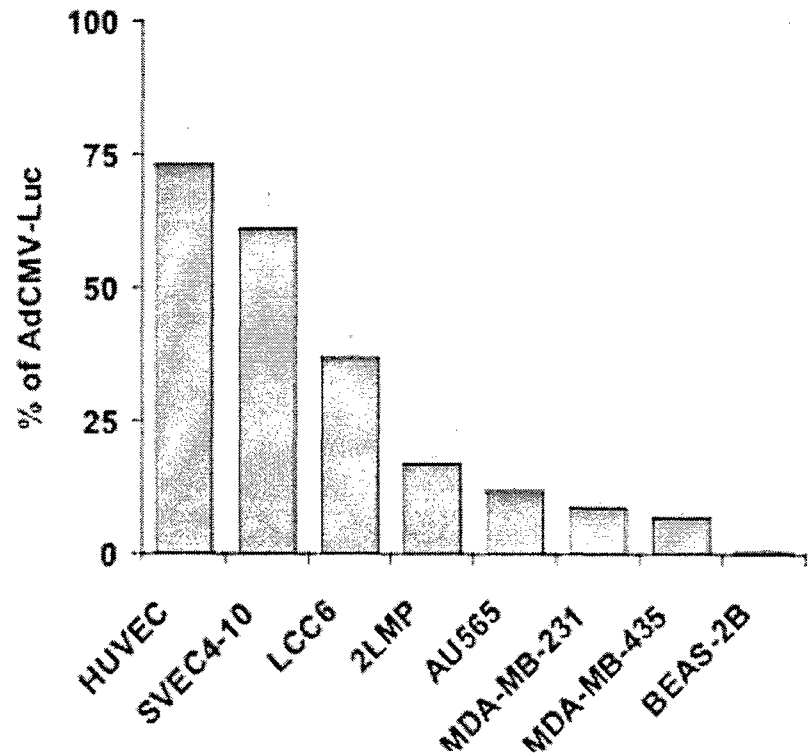

Fig. 1. Flt-1 promoter activity in human breast cancer cells, human and mouse endothelial cells. (A) Luc expression in breast cancer and endothelial cell lines. Human breast cancer cell lines, BEAS-2B normal human bronchial epithelial cells (negative control), SVEC 4-10 murine small vessel endothelial cells and HUVEC human umbilical vein endothelial cells (positive control) were infected with Adflt-Luc or AdCMV-Luc (control of infectivity) recombinant Ad at 50 MOI. Firefly luciferase expression was analyzed at 48 h after infection by luciferase assay system. Flt-1 promoter activity is presented as a percentage of CMV promoter activity. Presented are mean values ± SD of three independent experiments, each performed in triplicate.

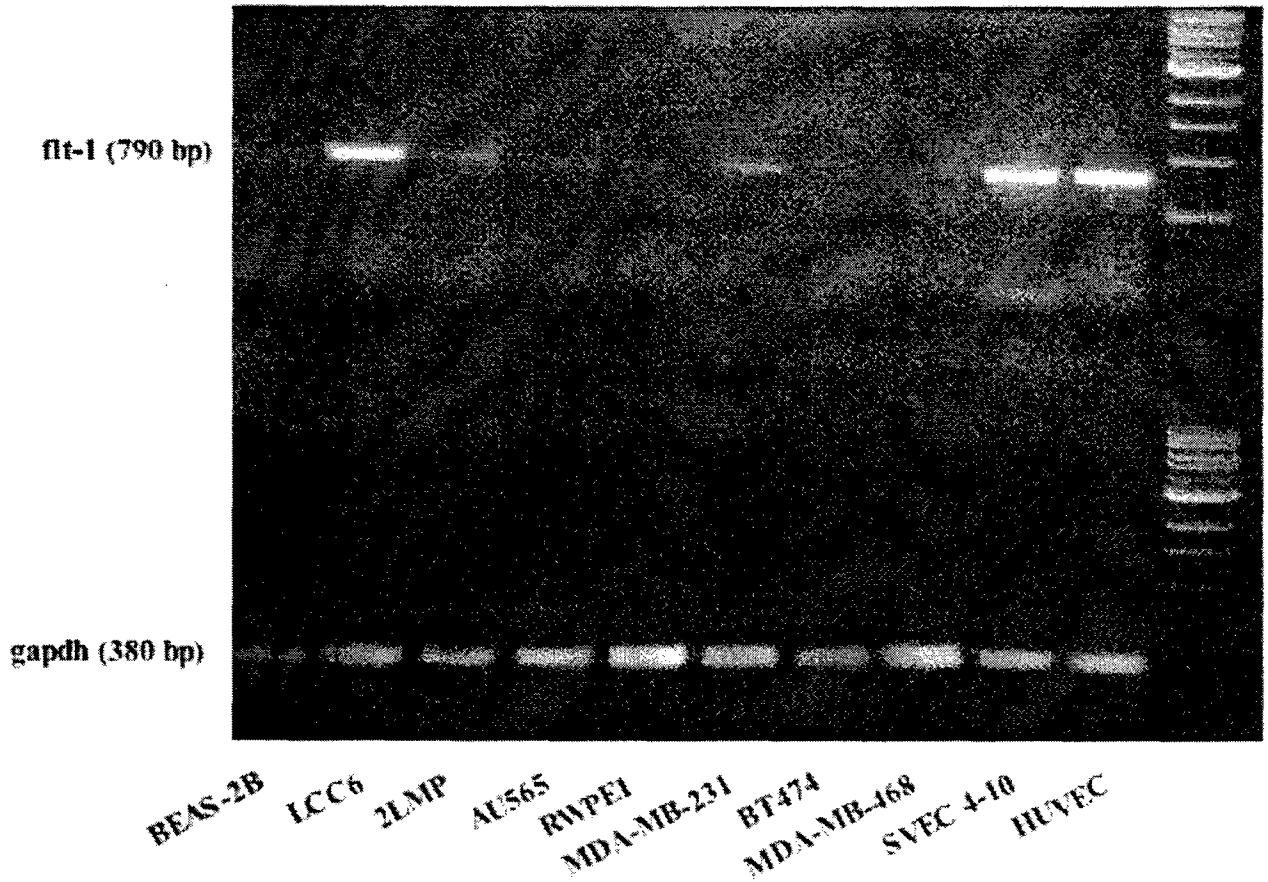

Fig. 1. Flt-1 promoter activity in human breast cancer cells, human and mouse endothelial cells. (B) The levels of *flt-1* mRNA in breast cancer cells and endothelial cells. BEAS-2B (negative control), breast cancer cells, SVEC 4-10 and HUVEC (positive control) were collected and total RNA was extracted. The levels of *flt-1* and *gapdh* (loading control) expression were determined using RT-PCR. One representative of three different experiments is shown.

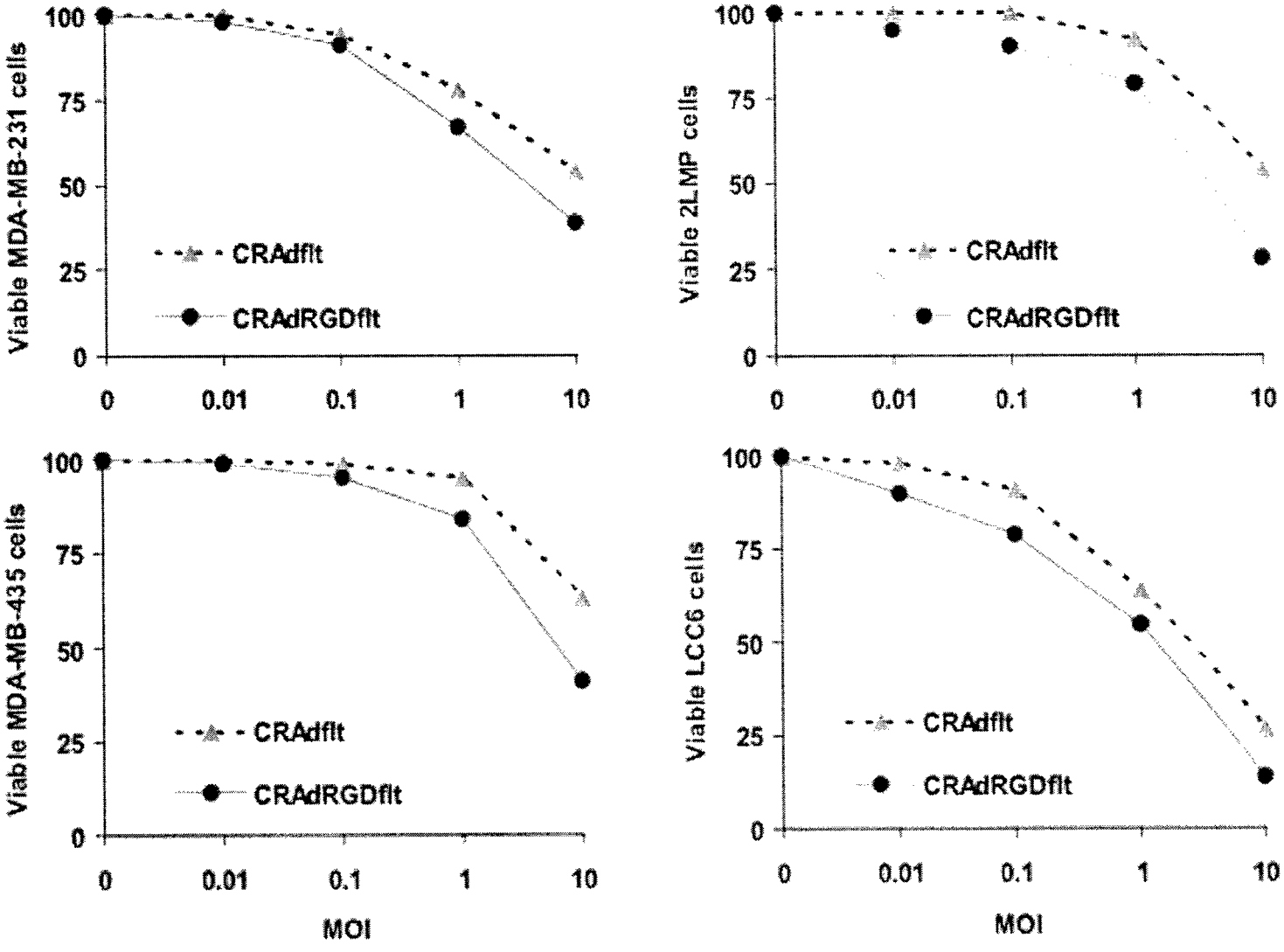

Fig. 2. RGD-4C modification of the Ad fiber-knob domain increases flt-1 driven CRAd oncolysis of breast cancer cells. Several breast cancer cell lines were infected with the CRAdflt or CRAdRGDflt at different MOI. Cell viability was determined at 96 h after infection by using the crystal violet inclusion assay. Data shown in comparison with uninfected control.

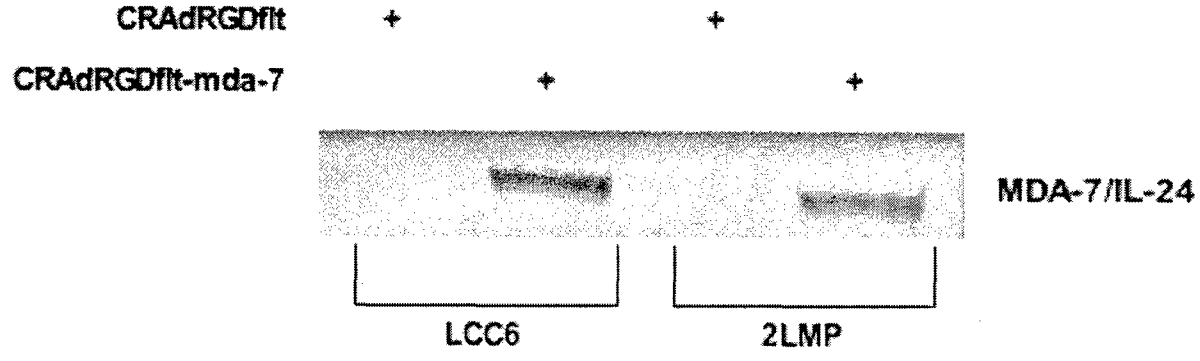

Fig. 3. *In vitro* CRAdRGDflt-mda-7 production. The level of expression of MDA-7/IL-24 protein in breast cancer cells. Protein lysate was collected from LCC6 and 2LMP cells infected with CRAdRGDflt-mda-7 or CRAdRGDflt (as negative control). The expression of the MDA-7/IL-24 protein was examined using a Western blotting analysis using polyclonal rabbit anti-mda-7/IL-24 antibody. Equal amounts (30 µg) of protein were loaded for each sample in all lanes and separated on SDS-PAGE followed by transfer to a PVDF membrane. One representative of three different experiments is shown.

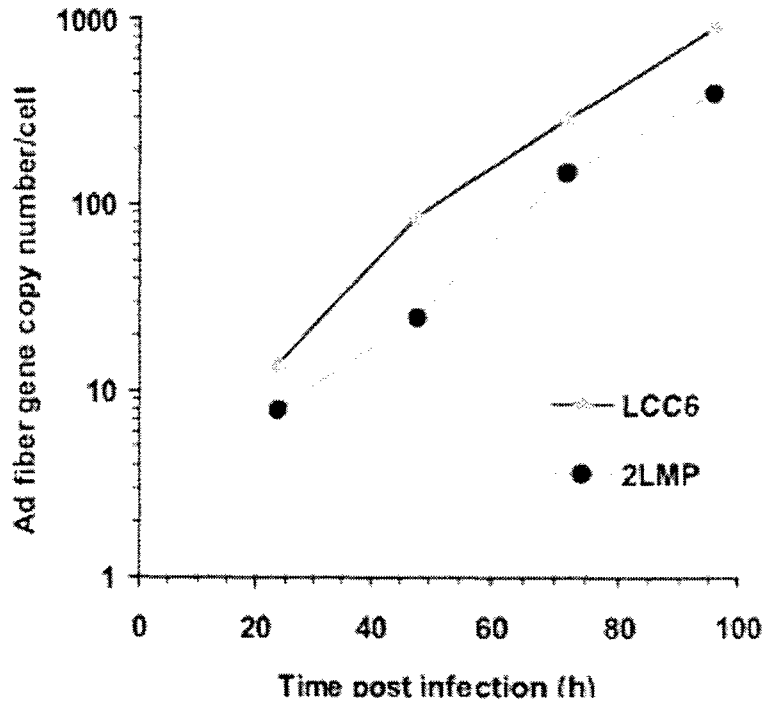

Fig. 4. *In vitro* CRAdRGDflt-mda-7 production. Ad fiber gene expression in CRAdRGDflt-mda-7-infected tumor cells. 2LMP and LCC6 breast cancer cells were infected with CRAdRGDflt-mda-7 at 1 MOI. Ad fiber gene copy number was determined by a Real-Time PCR assay at different times after infection. Relative Ad fiber gene copy number is expressed per cell. Values shown are mean values for triplicate samples.

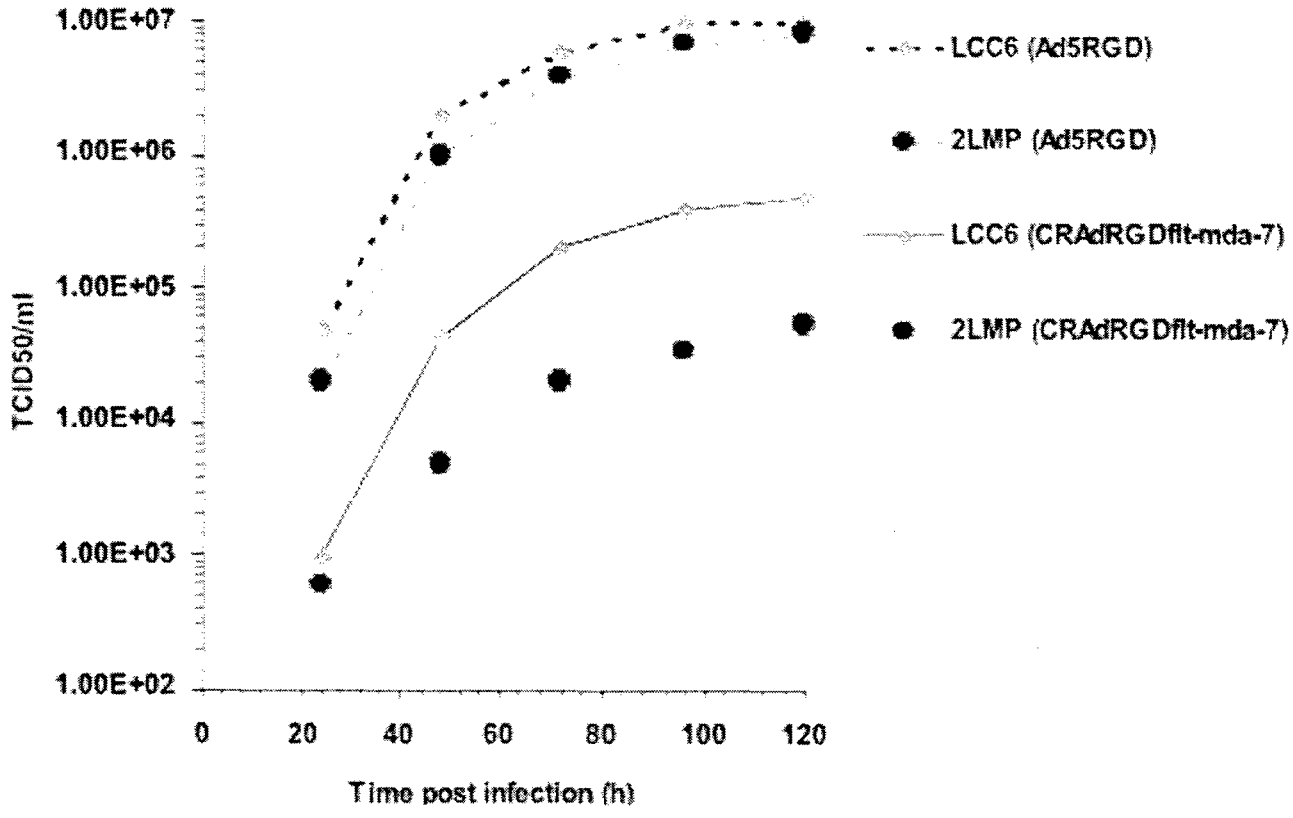

Fig. 5. *In vitro* CRAdRGDflt-mda-7 production. Replication efficiency of flt-1 driven CRAd in comparison with wt Ad5. Human breast cancer cells were infected with the CRAdRGDflt-mda-7 or Ad5RGD (wild type Ad5 with RGD-4C modification in fiber-knob domain) at 1 MOI. The virus titer was determined at a different time after infection by using the tissue culture infectious dose 50% ($TCID_{50}$) method.

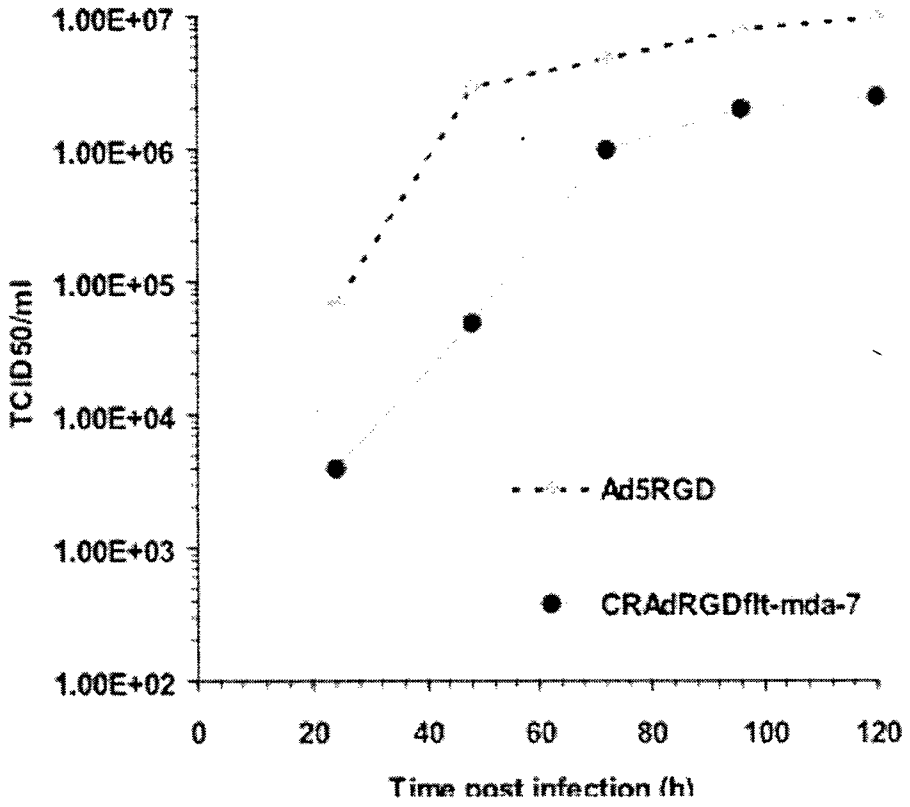

Fig. 6. *In vitro* CRAdRGDflt-mda-7 production in human blood vessel endothelial cells. HUVEC cells were infected with the CRAdRGDflt-mda-7 or Ad5RGD (wild type Ad5 with RGD-4C modification in fiber-knob domain) at 1 MOI. The virus titer was determined at a different time after infection by using the tissue culture infectious dose 50% ($TCID_{50}$) method.

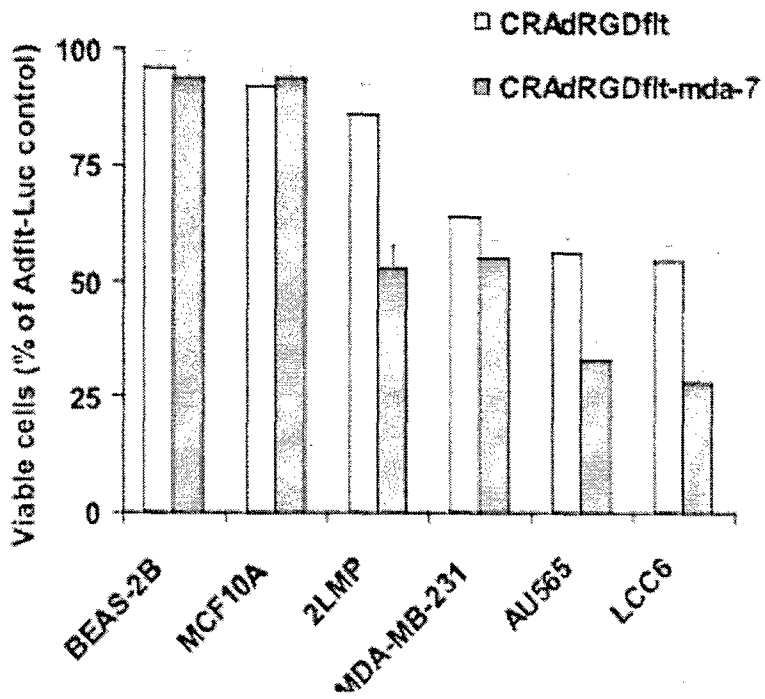

Fig. 7. CRAdRGDflt-mda-7 produces death in breast cancer cells. Human breast cancer cells, MCF10A normal mammary gland epithelial, and BEAS-2B normal human bronchial epithelial cells were infected with CRAdRGDflt-mda-7, CRAdRGDflt or Adflt-Luc at 1 MOI. Cell viability was determined at 96 h using the crystal violet staining assay. Data shown represent relative cell viability following CRAdRGDflt-mda-7 or CRAdRGDflt infection compared to Adflt-Luc. Presented are mean values ± SD of three independent experiments, each performed in six replicates.

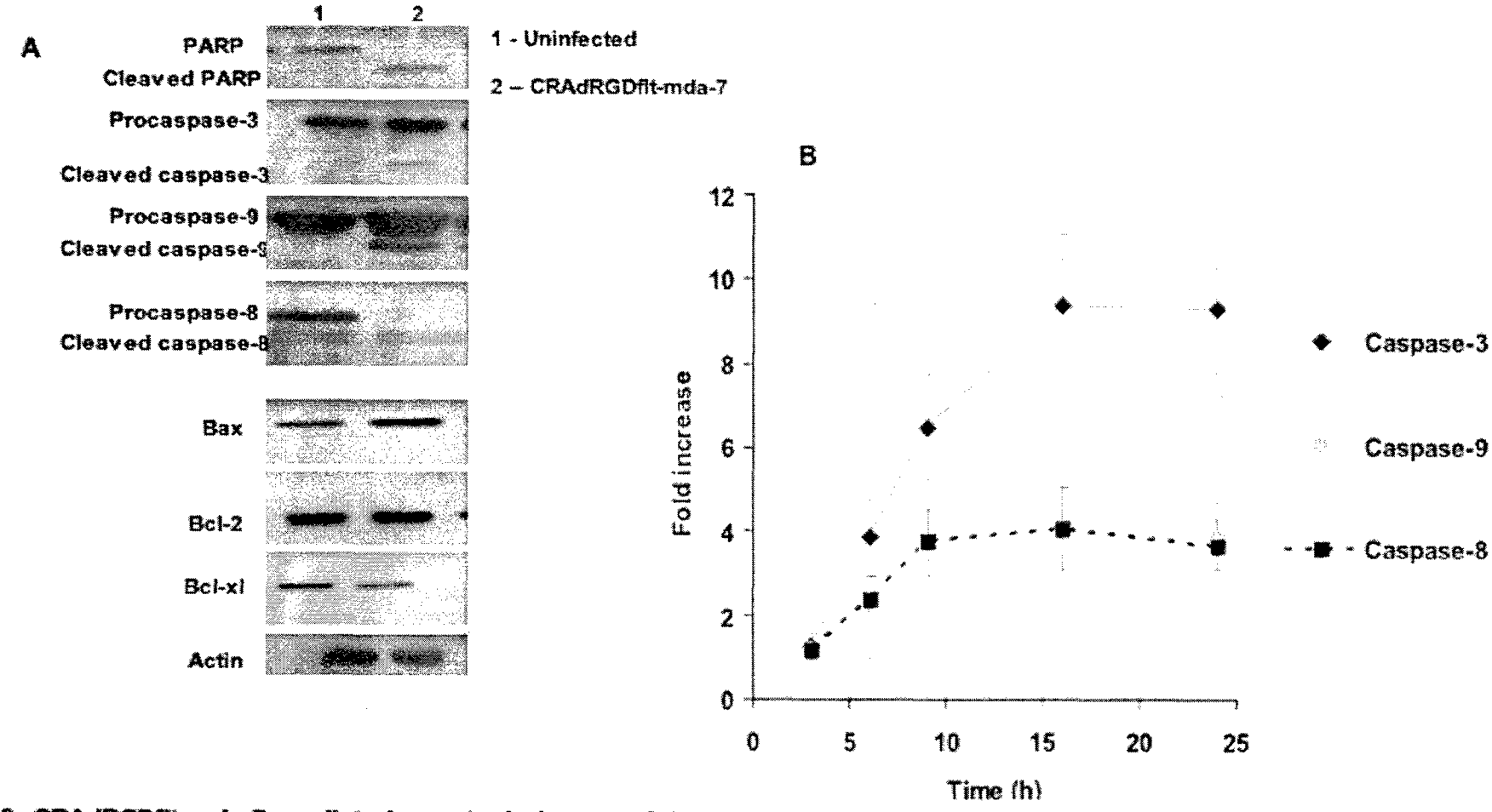

Fig 8. CRAdRGDflt-mda-7-mediated apoptosis is associated with caspase activation and regulation Bcl-2 family protein expression. (**A**) The cleavage of PARP and caspase-3,-8 and –9, and expression of Bax, Bcl-2, and Bcl-xl was monitored by Western blot analysis. LCC6 breast cancer cells were collected 16 h after CRAdRGDflt-mda-7 infection at 1 MOI. Equal amounts (30 μg) of protein were loaded for each sample in all lanes and separated on SDS-PAGE followed by transfer to a PVDF membrane. One representative of three different experiments is shown. (**B**) Caspase-like activity was measured using Ac-DEVD-AFC, Ac-IETD-AFC, and Ac-LEHD-AFC as fluorometric substrates for caspase-3,-8, and -9 respectively. After CRAdRGDflt-mda-7 infection (1 MOI), LCC6 breast cancer cells were harvested over time as indicated. Samples were read in a fluorometer equipped with a 400-nm excitation filter and 505-nm emission filter. Fold-increase in caspase activity was determined by comparing result with the level of the uninfected control cells. Data shown are the mean ± SD.